IV．内科医がみる脳卒中

KeyWords
◎一過性脳虚血発作
◎脳梗塞
◎リスク評価
◎ABCD²スコア
◎脳卒中ガイドライン2009

一過性脳虚血発作の診断と治療

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Headline

1. 一過性脳虚血発作（TIA）という病態概念はここ数年で劇的に変化してきている．
2. TIAの初期診断では，病歴から局所神経症状の出現を正しくとらえることが重要である．
3. TIAと診断，または疑われる場合は直ちに専門病院へ送る必要がある．
4. TIAの治療はリスク評価を行い，発症機序に基づいて行う．
5. TIAは“急性脳血管症候群”として迅速，適正に扱うべきである．

はじめに

一過性脳虚血発作(transient ischemic attack；TIA）とは一般的に，様々な機序により脳局所の血管の灌流が急激に悪化し，半身の麻痺や構音障害などの症状が短期間生じるが，脳組織に損傷をきたすことなく回復する脳虚血発作である．TIAは脳梗塞の前触れ，警告発作として重要であり，その早期発見・早期治療が最も大切であるとされているが，専門外の医師にとっては外来で経過をみる一時的で良性の神経症候群の総称と認識されている例が少なくない．また，TIAが重大な警告発作（緊急症）と認識しつつも，ほとんどの例が診察時には症状が改善しているため，直ちに初期評価を行い，迅速に治療する施設も多くはない現状であった．

しかし，TIAに対する認識はここ数年で劇的に変化してきた．TIAは短期間に続発する脳梗塞の前兆発作，将来の心血管イベントのリスク因子としても重要視されており，最近は危険なTIAのリスク分析を行い，早期から積極的な心血管評価と治療を行うことで，その後の脳梗塞発症を予防しうることが強調されている[1,2]．このほど，TIAの新しい定義として提唱されたtissue-based TIAを是認する科学コメントがアメリカ心臓協会/アメリカ脳卒中協会(American Heart Association/American Stroke Association；AHA/ASA）より発表された[3]．新しい定義ではTIAの診断基準を症状持続時間で区切ることはあまり意味がないと考え，“脳，脊髄，網膜の局所の虚血による短時間の神経学的な機能障害で，画像診断で脳梗塞を認めないもの”と定義されており，今後，世界的なコンセンサスになっていくことが予想される．

本稿ではTIAに関する初期対応と症候からの診断，それに基づいた治療について解説する．

診断

TIAの初期診断において最も重要なことは，局所神経症状の出現を正しくとらえることである．TIAの症状の多くは短時間（数分～数十分）で消失しているため，詳細な病歴聴取が大変重要となってくる．診察時の頸部血管雑音，血圧の左右差，不整脈の存在はTIAに随伴しうる所見として診断の助けとなる．

0370-999X/11/¥100/頁/JCOPY

Neuroscience Letters 495 (2011) 210–215

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Gene and protein analysis of brain derived neurotrophic factor expression in relation to neurological recovery induced by an enriched environment in a rat stroke model

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ARTICLE INFO

Article history:
Received 22 October 2010
Received in revised form 7 March 2011
Accepted 22 March 2011

Keywords:
Focal ischemia
Enriched environment
Brain derived neurotrophic factor

ABSTRACT

Although an enriched environment enhances functional recovery after ischemic stroke, the mechanism underlying this effect remains unclear. We previously reported that brain derived neurotrophic factor (BDNF) gene expression decreased in rats housed in an enriched environment for 4 weeks compared to those housed in a standard cage for the same period. To further clarify the relationship between the decrease in BDNF and functional recovery, we investigated the effects of differential 2-week housing conditions on the mRNA of BDNF and protein levels of proBDNF and mature BDNF (matBDNF). After transient occlusion of the right middle cerebral artery of male Sprague–Dawley rats, we divided the rats into two groups: (1) an enriched group housed multiply in large cages equipped with toys, and (2) a standard group housed alone in small cages without toys. Behavioral tests before and after 2-week differential housing showed better neurological recovery in the enriched group than in the standard group. Synaptophysin immunostaining demonstrated that the density of synapses in the peri-infarct area was increased in the enriched group compared to the standard group, while infarct volumes were not significantly different. Real-time reverse transcription polymerase chain reaction, Western blotting and immunostaining all revealed no significant difference between the groups. The present results suggest that functional recovery cannot be ascribed to an increase in matBDNF or a decrease in proBDNF but rather to other underlying mechanisms.

Functional impairment caused by stroke is a highly serious health problem throughout the world. Rehabilitation has been widely applied and has been shown to contribute greatly to neurological recovery. However, the mechanisms of the beneficial effects of rehabilitation remain unclear [3]. An enriched environment is a model of rehabilitation for rodents, in which multiple animals are housed together in a large cage equipped with toys. Enriched environments have been shown to enhance the recovery of neurological function impaired by experimental focal ischemia [13]. Brain-derived neurotrophic factor (BDNF), one of the neurotrophins, may be a key molecule in this effect, since it is central to many facets of the neural network, from differentiation and neuronal survival to synaptogenesis and activity-dependent forms of synaptic plasticity [9]. While an enriched environment increases BDNF expression in non-ischemic healthy animals [5], this is not the case with ischemic animals. The alteration of BDNF after ischemic stroke is not fully understood, although BDNF expression has been investigated in association with an enriched environment after experimental stroke. Zhao et al. demonstrated that BDNF mRNA

Abbreviations: BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPT, inclined plane test; MAP-2, microtubular-associated protein 2; matBDNF, mature BDNF; MCA, middle cerebral artery; NSS, neurological severity scores; ROI, region of interest; RT-PCR, reverse transcription polymerase chain reaction; SYP, synaptophysin; tMCAO, transient MCA occlusion.

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doi:10.1016/j.neulet.2011.03.068

[19] and BDNF protein [20] were decreased after ischemic stroke and housing in an enriched environment. Nygren et al. reported that BDNF +/− mice, which express low levels of BDNF, showed better stroke recovery in an enriched environment than their wild-type counterparts [12]. On the other hand, Risedal et al. observed no significant change of BDNF mRNA between rats in an enriched environment and those in a standard environment in their experiments using a permanent occlusion model [15]. In our previous investigation, microarray analysis and real-time reverse transcription polymerase chain reaction (RT-PCR) revealed a significant decrease in expression of the BDNF gene in the contralateral cortex to ischemia in rats in 4-week enriched environment [16]. Generally, BDNF is a beneficial molecule for neurons and neurological functions, and there thus appears to be a discrepancy between the decreased BDNF and improved neurological functions in these studies. There are at least two possible explanations for this phenomenon. The first one is that BDNF may be down-regulated, since the functionally improved brain may no longer need elevated BDNF after 4-week enrichment. Another possibility is that BDNF itself is an exacerbating factor for neurological deficit, especially in the post-stroke state, and that an enriched environment may eliminate BDNF to avoid its potentially deleterious effects. To examine these hypotheses, it will be necessary to examine brain tissue at an earlier time point when functional recovery has not been completed. Furthermore, proBDNF, a precursor of the BDNF protein, must be investigated, since it negatively influences neurons [9]. Thus, the objective of the present study was to investigate the expression of BDNF in rats subjected to focal cerebral ischemia followed by housing for 2 weeks in an enriched environment by using RT-PCR, Western blotting, and immunohistochemical techniques.

Nine-week-old, male Sprague–Dawley rats were anesthetized by chloral hydrate and the right middle cerebral artery (MCA) was occluded intraluminally for 60 min with nylon monofilaments, as previously described [8]. At 72–96 h after transient MCA occlusion (tMCAO), the rats were randomly divided into two groups, an enriched group and a standard group. For the enriched group, 4–6 rats were housed together in a large cage (610 mm × 460 mm × 460 mm) containing toys including a running wheel, a tunnel, balls, logs and rings, rearranged twice a week. For the standard group, rats were housed alone in a standard-sized cage (320 mm × 210 mm × 130 mm) containing food and water.

Ischemic animals were subjected to two behavioral tests, the neurological severity score test (NSS) [2] and the inclined plane test (IPT) [6]. These tests were performed 3 times, once before tMCAO, once at 3 or 4 days after tMCAO (defined as day 0), and once at 14 days after initiation of differential housing (defined as day 14). The NSS is a composite of motor, sensory, reflex, and balance tests. The score ranges from 0 to 18, with the higher score indicating severe neurological impairment. In this study, we analyzed rats that scored between 7 and 12 in the second test (day 0). The recovery rate was defined as $(NSS_{2nd} - NSS_{3rd})/NSS_{2nd}$. The IPT was performed to evaluate motor deficits. Each rat was placed up-headed or right-headed on a stainless steel plane steepening at a rate of $2^\circ\,s^{-1}$, and we recorded the angle when the rat slipped on the plate. The improvement index was calculated as $(IPT_{3rd} - IPT_{2nd})/(IPT_{1st} - IPT_{2nd})$. After the behavioral tests on day 14, rats were sacrificed and the brains were cut into 3 coronal sections with a thickness of 3 mm from the frontal pole.

The second blocks from the frontal pole were embedded in paraffin for the histological study. Microtubular-associated protein 2 (MAP-2), glial fibrillary acidic protein (GFAP), synaptophysin (SYP), and matBDNF were immunohistochemically stained. The infarct volume was calculated as $(C - I)/C$, where C represents MAP-2-stained volume in contralateral side, and I represents MAP-2-stained volume in ischemic side. To set regions of interest in peri-infarct area, we assessed both neuronal viability using MAP-2 staining and glial activity using GFAP staining. MAP-2 mainly distinguishes infarct area from non-infarct area, and GFAP mainly distinguishes peri-infarct area and distant intact area. The area with both preserved MAP-2 staining and intense GFAP staining was defined as peri-infarct area. SYP and BDNF immunoreactivity was quantified in peri-infarct area and its contralateral cortex. The rate of the positively stained area was compared between the two groups.

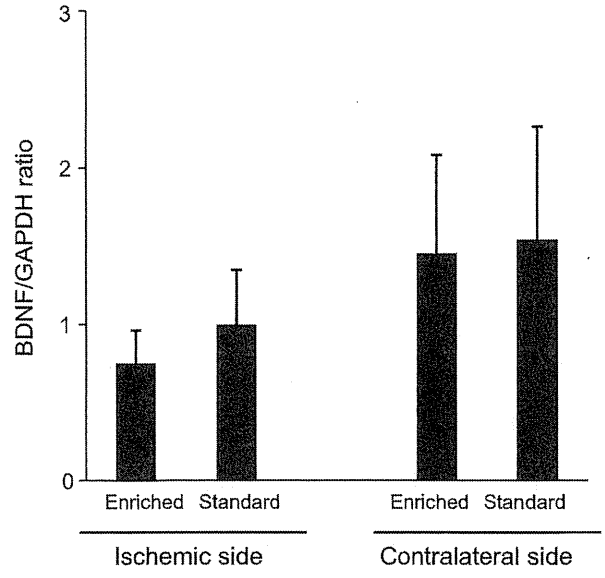

Fig. 1. BDNF gene expression showed no significant differences between the enriched and standard groups on the ischemic side ($p = 0.16$) or contralateral side ($p = 0.81$). Data were normalized to the ischemic side of the standard group.

The peri-infarct cortex and its contralateral cortex of the third blocks were subjected to real-time RT-PCR or Western blotting. Total RNA from the peri-infarct cortex and contralateral cortex was isolated and analyzed for gene expression by real-time quantitative RT-PCR. Expression levels of BDNF mRNA were normalized to those of GAPDH mRNA. For Western blotting, primary antibodies were HRP-conjugated anti-β actin antibody, anti-BDNF antibody, and anti-proBDNF antibody. The anti-proBDNF antibody was produced in a rabbit by intravenous injection of proBDNF-specific peptide. The secondary antibody was HRP-conjugated anti-rabbit goat IgG. The chemiluminescence agents were ECL or ECL+Plus (GE Healthcare). An LAS-4000miniEPUV (FUJIFILM) CCD camera was used to quantify the band intensity. As positive controls, recombinant human BDNF and C6 glioma cell lysate was applied for Western blotting.

Data are expressed as the means ± SD, and a p-value less than 0.05 was considered statistically significant. See the supplementary document for more information about the methods.

The neurological and motor functions of rats in both groups were impaired after t-MCAO and improved on day 14 (Table 1). A significant difference was observed in both NSS and IPT on day 14, but not on day 0, between the enriched ($n = 24$) and standard ($n = 22$) groups. The recovery rate for NSS and improvement index for IPT both indicated a significant improvement in function in the enriched group compared to the standard group.

The infarction area evaluated by immunoreactivity to MAP-2 in the enriched group (56.82 ± 7.31%, $n = 14$) was not significantly different from that in the standard group (55.44 ± 11.50%, $n = 13$, $p = 0.72$).

We performed real-time RT-PCR to examine the changes in BDNF levels (Fig. 1). The data presented were normalized to the ischemic side of the standard group. On the ischemic side, the BDNF/GAPDH ratio was 0.75 ± 0.21 ($n = 7$) in the enriched group, which was slightly but not significantly lower than that in the stan-

Table 1
Behavioral test results.

	Pre-MCAO		Day 0		Day 14	
	Enriched $n=24$	Standard $n=22$	Enriched	Standard	Enriched	Standard
NSS	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 1.02	7.55 ± 0.74	4.92 ± 1.32*	5.73 ± 1.03
NSS recovery rate					37.83 ± 17.95*	23.67 ± 14.08
IPT up-headed	51.72 ± 2.00	50.80 ± 1.74	42.26 ± 2.61	43.52 ± 2.34	47.54 ± 1.63*	45.37 ± 2.30
IPT right-headed	50.60 ± 2.86	50.79 ± 2.62	41.63 ± 2.62	42.80 ± 2.94	47.08 ± 2.11*	45.61 ± 2.11
IPT mean	51.16 ± 2.16	50.79 ± 1.90	41.94 ± 2.39	43.16 ± 2.37	47.31 ± 1.70*	45.49 ± 2.15
IPT improvement index					59.17 ± 29.51*	27.75 ± 40.25

* $p<0.05$ compared to the standard group. Data are presented as the means ± SD.

dard group (1.00 ± 0.35, $n=6$). On the contralateral non-ischemic side, there was no significant difference in the BDNF/GAPDH ratio between the enriched group (1.45 ± 0.63, $n=8$) and standard group (1.54 ± 0.72, $n=6$).

Fig. 2 summarizes the results of Western blotting for matBDNF and proBDNF. The antibodies for matBDNF and proBDNF were validated with Western blotting using recombinant matBDNF and C6 glioma cell lysate, respectively (Fig. 2A). There were no significant differences between the enriched group ($n=8$) and standard group ($n=8$) in either matBDNF on the ischemic side (Fig. 2B), matBDNF on the contralateral side (Fig. 2C), proBDNF on the ischemic side (Fig. 2D), or proBDNF on the contralateral side (Fig. 2E), although the level of matBDNF in ischemic side tended to be slightly lower in the enriched group than in the contralateral group.

Fig. 3 shows the results of immunohistochemical staining of GFAP, SYP and BDNF. The areas stained with SYP and BDNF were quantified. The SYP-stained area on the ischemic side ($n=14$) was significantly increased in the enriched group ($n=13$) compared to the standard group (Fig. 3B, 2.57 ± 0.28% vs 2.07 ± 0.23%, $p<0.001$), although no significant difference was observed on the contralateral side (Fig. 3C, 1.56 ± 0.29% vs 1.49 ± 0.30%, $p=0.52$). On the other hand, the matBDNF-stained area on the ischemic side was slightly smaller in the enriched group ($n=15$) than in the standard group ($n=12$), although the difference did not reach the level of statistical significance (Fig. 3D). On the contralateral side, the enriched group showed a matBDNF-stained area comparable to that of the standard group without significant difference (Fig. 3E).

This study showed that housing in an enriched environment for 2 weeks significantly enhanced the functional recovery of rats after ischemic stroke. In addition, the immunohistochemical findings of increased SYP staining indicated an increased density of synapses. On the other hand, no significant difference was observed in the volume of infarction, mRNA expression of BDNF, or protein expressions of BDNFs.

Our previous investigation using a 4-week period of housing demonstrated a decrease in BDNF gene in the animals housed in an enriched environment based on microarray analysis and real-time RT-PCR as well as a continuous improvement of neurological functions until 4-week [16]. To further clarify the mechanisms of decreased BDNF expression, in the present study we measured the levels of the BDNF protein and gene after a shorter period of enriched environment, i.e., 2 weeks. The common finding between

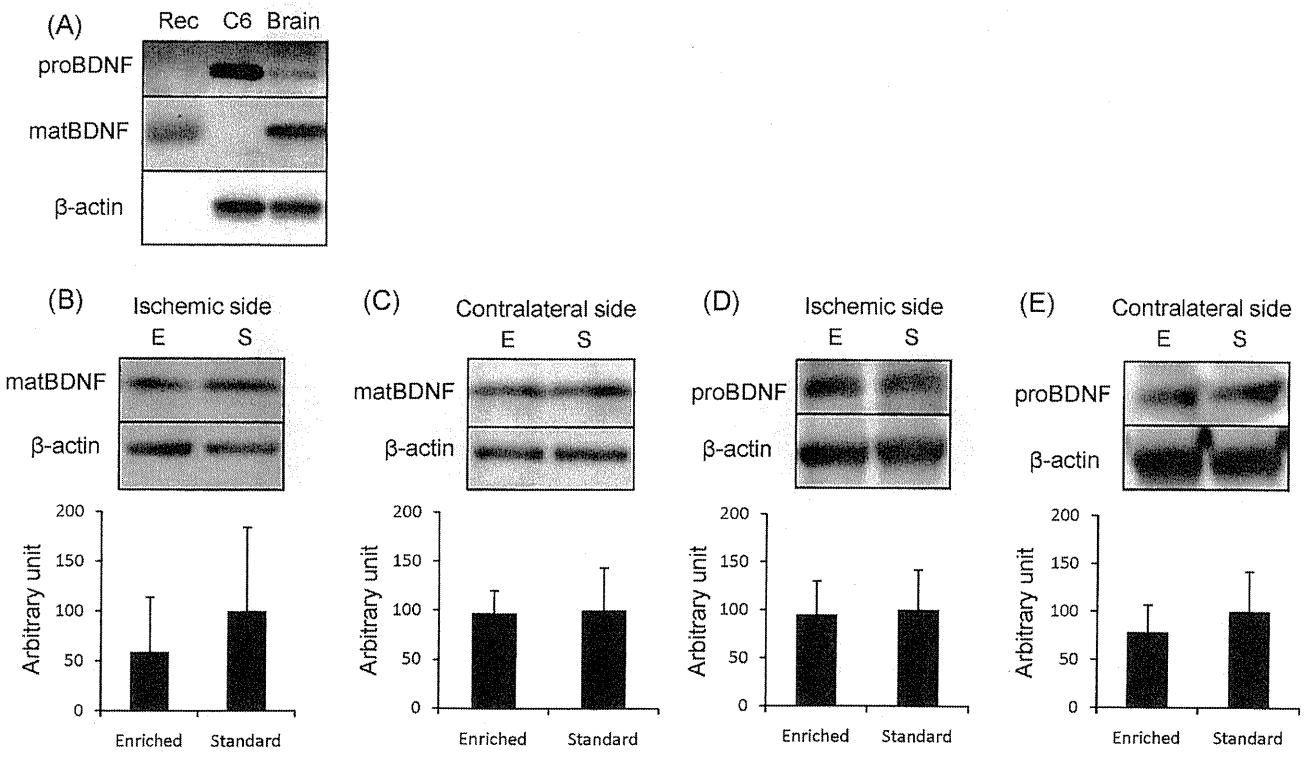

Fig. 2. Western blotting. (A) Recombinant human matBDNF (Rec), C6 glioma cell lysate (C6), and rat brain tissue validated the use of the antibodies for matBDNF (14 kDa) and proBDNF (32 kDa). Rat brain tissue contained detectable levels of these molecules. The levels of (B) matBDNF on the ischemic side ($p=0.27$), (C) matBDNF in contralateral side ($p=0.86$), (D) proBDNF on the ischemic side ($p=0.79$), and (E) proBDNF on the contralateral side ($p=0.25$) were not significantly different between the enriched and standard groups. Abbr. E: enriched group; S: standard group.

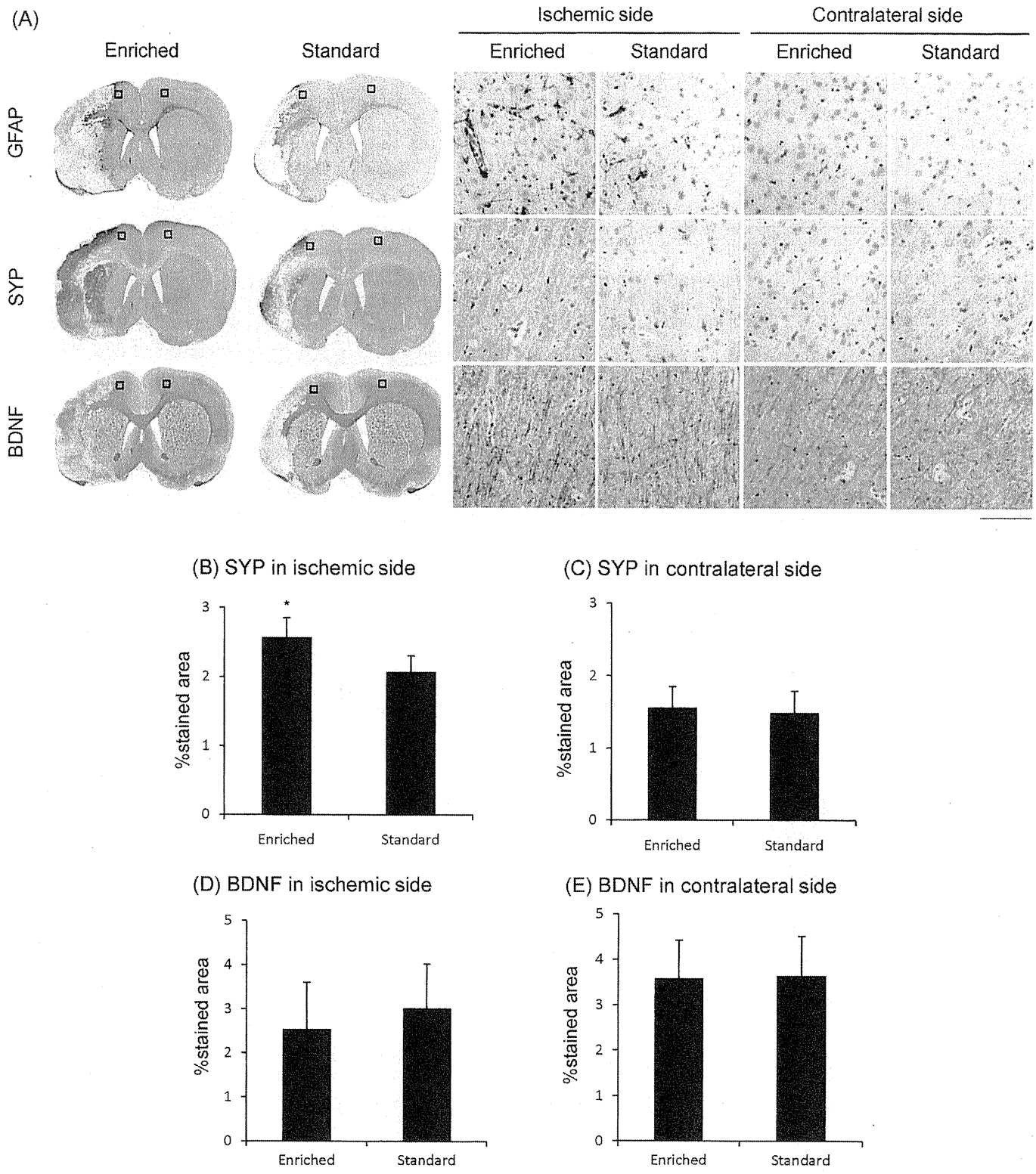

Fig. 3. Immunohistochemical analysis of GFAP, SYP, and matBDNF levels on the ischemic side and contralateral side. (A) Representative whole brain images (left 2 columns) and enlarged images (right 4 columns). The ischemic side was the left in the picture (right hemisphere of the rats). Squares indicate the enlarged sites. The bar represents 100 μm. (B–E) The SYP- and BDNF-stained areas were quantified. The ischemic side in the enriched group showed significantly stronger staining than the ischemic side in the standard group (B) ($p < 0.001$), while the contralateral side in the enriched group and contralateral side in the standard group did not show a significant difference (C) ($p = 0.52$). On the other hand, the matBDNF-stained area on the ischemic side (D) ($p = 0.24$) and contralateral side (E) ($p = 0.85$) showed no significant difference.

the 2-week and 4-week experiments was the lack of a clear increase in BDNF gene expression, despite the well-known beneficial effects of BDNF for neurons and neurological functions under various physiological and pathological conditions. These results, combined with our previous findings showing amelioration of neurological sign after 2 weeks, suggest that the functional recovery induced by an enriched environment might be brought about by mechanisms other than increased BDNF.

As an explanation for the relationship between BDNF and functional recovery, we hypothesized that proBDNF might play an

important role. The primary product of the BDNF gene is the 32-kDa proBDNF protein, which is translated in neurons and released into the extracellular space. Proteolytic enzymes such as plasmin cleave proBDNF into 14-kDa matBDNF and another particle. While matBDNF binds to the TrkB receptor on neurons and induces cell survival, differentiation, and long-term potentiation, proBDNF binds to the p75NTR receptor on neurons and induces apoptosis of neurons and long-term depression [9,11,17]. Thus, proBDNF and matBDNF, originating from the same gene, have opposite effects. Therefore, not only transcriptional regulation but also post-translational modification must be taken into consideration when we discuss the effects of BDNF. Via its pro-apoptotic effect, proBDNF might have an exacerbating effect on the ischemic damage of neurons and therefore neurological functions. If so, the decrease in proBDNF is beneficial. However, to our knowledge, there has been no studies investigating proBDNF expression in relation to stroke and enriched environment, although Zhao et al. previously measured the total levels of matBDNF and proBDNF using ELISA with anti-matBDNF antibody [20]. In the present work, we first tried to measure proBDNF using commercially available antibodies. Theoretically, Western blotting using either a proBDNF-specific antibody or matBDNF antibody could be used to quantify proBDNF levels, since the matBDNF domain is common to proBDNF and matBDNF. However, we did not find an efficient antibody that clearly revealed the band of proBDNF. Therefore, in the present study, we produced a polyclonal antibody using a proBDNF-specific peptide. Contrary to our expectations, Western blotting revealed no significant difference in proBDNF expression between the enriched group and standard group. This finding clearly indicates that the functional improvement induced by an enriched environment cannot be ascribed to decreased proBDNF.

On the other hand, an interesting difference between the 2-week and 4-week experiment is that a significant decrease in BDNF gene expression was observed after 4 weeks but not after 2 weeks of enrichment. Therefore, the difference in BDNF expression may be amplified between 2 weeks and 4 weeks. Considering that functional recovery and synaptogenesis were already observed at 2 weeks, there is a possibility that the decrease of BDNF expression at 4 weeks might result from secondary down-regulation due to the improved neurological functions. To prove the hypothesis, further investigation will be necessary to collect samples in earlier time points than 2 weeks, especially before the significant improvement becomes evident in enriched group. Not only BDNF gene expression but also matBDNF protein showed a consistent trend of decrease by both Western blotting and immunohistochemical staining, although the difference was not statistically significant. These results agree with the report by Zhao et al., which demonstrated a decrease in the total levels of BDNF protein in an enriched group [20]. Thus, we cannot completely rule out type-2 statistical error that small analyzing numbers concealed the possible decrease in BDNF genes or matBDNF in enriched group. Even if that is the case, however, there is no evidence of increase in BDNF genes or matBDNF.

Few genes are known to have a relationship to enriched housing environments. Dahlqvist et al. reported an alteration in the levels of nerve growth factor-induced gene A and glucocorticoid receptor following environmental enrichment in rats [4]. We previously found that animals in an enriched housing group showed a decrease in Early growth response-1 (Erg-1) mRNA [16], which is an inflammatory gene associated with exacerbation of neurological deficits after stroke [18]. To clarify the mechanisms of enriched environment-induced functional recovery, future investigations of neurotrophic factors other than BDNF (i.e., nerve growth factor, neurotrophin-3, and neurotrophin-4) and other inflammatory genes will be needed. Since the environmental stimulation involves a series of complicated processes through which many genes and proteins are expected to alter their expression, the combination of exhaustive analysis of the relevant genes and proteins will provide additional information.

We observed no decrease in infarction volume in the enriched group. This finding is consistent with previous reports [1,13]. Functional recovery induced by an enriched environment is not due to a reduction of infarct volume but possibly due to functional compensation in the brain tissues that escaped from infarction. The increased density of synapses in peri-infarct cortex measured using the SYP-stained area further supports this hypothesis.

One may argue that an enriched environment is a model enhancing voluntary exercise and thus the effect depends on individual activity, leading to a great individual variability in stroke recovery. However, as far as animal experiments are concerned, the effect of forced exercise such as treadmill running is controversial [7,10,14], while most studies on environmental enrichment agree on its positive effects. Therefore, we consider that enriched housing is a more feasible model to investigate the mechanisms of functional recovery after brain ischemia. Because it is established that environmental enrichment leads to an increase in BDNF in non-ischemic healthy animals [5], we compared only ischemic rats between enriched and standard environments according to the previous research designs [19,20].

In conclusion, an improvement of neurological functions was induced by an enriched environment accompanied with an increased density of synapses but without a reduction of infarct volume. The 2-week environmental enrichment did not significantly alter BDNF expression, including BDNF mRNA, matBDNF protein, or proBDNF protein. These results suggest that the functional recovery might not be due to increased BDNF or decreased proBDNF but rather to other underlying mechanisms.

Acknowledgements

This study was partially supported by Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. This research was also partially supported by a Grant-in-Aid for General Scientific Research from the Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.03.068.

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Prospective multicentre cohort study of heparin-induced thrombocytopenia in acute ischaemic stroke patients

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Summary

Acute ischaemic stroke patients sometimes receive heparin for treatment and/or prophylaxis of thromboembolic complications. This study was designed to elucidate the incidence and clinical features of heparin-induced thrombocytopenia (HIT) in acute stroke patients treated with heparin. We conducted a prospective multicentre cohort study of 267 patients who were admitted to three stroke centres within 7 d after stroke onset. We examined clinical data until discharge and collected blood samples on days 1 and 14 of hospitalization to test anti-platelet factor 4/heparin antibodies (anti-PF4/H Abs) using an enzyme-linked immunosorbent assay (ELISA); platelet-activating antibodies were identified by serotonin-release assay (SRA). Patients with a 4Ts score ≥4 points, positive-ELISA, and positive-SRA were diagnosed as definite HIT. Heparin was administered to 172 patients (64·4%: heparin group). Anti-PF4/H Abs were detected by ELISA in 22 cases (12·8%) in the heparin group. Seven patients had 4Ts ≥ 4 points. Among them, three patients (1·7% overall) were also positive by both ELISA and SRA. National Institutes of Health Stroke Scale score on admission was high (range, 16–23) and in-hospital mortality was very high (66·7%) in definite HIT patients. In this study, the incidence of definite HIT in acute ischaemic stroke patients treated with heparin was 1·7% (95% confidence interval: 0·4–5·0). The clinical severity and outcome of definite HIT were unfavourable.

Keywords: acute stroke care, anticoagulation, heparin, platelet, thrombocytopenia.

Received 10 March 2011; accepted for publication 15 May 2011

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Immune-mediated heparin-induced thrombocytopenia (HIT), which is caused by platelet-activating IgG antibodies that recognize platelet factor 4 bound to heparin (anti-PF4/heparin Abs), is a relatively common side effect of heparin therapy and presents a strong risk factor for thromboembolic events associated with high mortality and morbidity (Warkentin, 2007a). Prospective studies in Western countries have shown that the prevalence of HIT is 0·3–5% of patients treated with unfractionated heparin (UFH), which varies depending on the clinical settings (Warkentin *et al*, 1995, 2000; Kappers-Klunne

First published online 14 June 2011
doi:10.1111/j.1365-2141.2011.08775.x

et al, 1997). Thrombotic complications occur in approximately one-third to one-half of HIT patients (Warkentin, 2007a). On the other hand, some studies of UFH therapy for acute stroke reported no cases of HIT (Toth & Voll, 2002; Camerlingo *et al*, 2005). To elucidate the prevalence of HIT in acute ischaemic stroke patients who were treated with heparin, we organized a prospective multicentre cohort study that included systematic collection of blood for detection of the antibodies that cause HIT.

Some clinical guidelines do not recommend prescribing heparin in acute ischaemic stroke, and others recommend it mainly for the prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE) (Albers *et al*, 2004; Cardiovascular Disease Educational and Research Trust, 2006; Adams *et al*, 2007). At the participating stroke centres in our study, in addition to the prevention of DVT and PE, UFH is given during the acute phase of ischaemic stroke to the following: patients with emboligenic heart disease or superimposed thrombi on the carotid plaque to prevent embolic complications; patients with particular stroke aetiologies, including cerebral arterial dissection and vasculitis; and patients with embolic stroke of unknown origin until the presence of heart disease is excluded by the results of prolonged electrocardiography and transesophageal echocardiography (Caplan, 2003).

In a previous study of 137 stroke patients who were treated with UFH, 21 patients (15·3%) developed thrombocytopenia (≥40% fall in platelet counts) during or after heparin therapy, and five of these 21 patients had an additional ischaemic stroke (Ramirez-Lassepas *et al*, 1984). A recent study of 200 neurological patients treated with UFH for at least 5 d, including 102 patients with cerebrovascular disorders, demonstrated that 41 patients (20·5%) had anti-PF4/heparin Abs and 5 (2·5%) developed HIT, when the serological diagnosis was made from the presence of antibodies detected by an enzyme-linked immunosorbent assay (ELISA) (Harbrecht *et al*, 2004).

Only a few studies have investigated the prevalence of HIT in acute stroke patients receiving UFH, especially in the Asian population (Kawano *et al*, 2008). In our previous retrospective report of acute ischaemic stroke patients who were treated with UFH, 0·5% of the patients developed HIT diagnosed by both the clinical scoring systems and the serological assays, including ^{14}C-serotonin release assay (SRA) (Kawano *et al*, 2008). However, our retrospective study assessing the prevalence of HIT was limited by the fact that was that antibodies were not assayed in all patients. This limitation may cause an under diagnosis of HIT.

Thus, we performed this prospective multicentre cohort study in 267 patients to determine a more accurate incidence of HIT in patients with acute ischaemic stroke and to elucidate the clinical features of HIT.

Methods

Study design

A prospective multicentre cohort study.

Subjects and settings

This study was conducted in three Japanese stroke centres at the then National Cardiovascular Centre (currently the National Cerebral and Cardiovascular Centre, Osaka), Research Institute for Brain and Blood Vessels Akita (Akita), and Kumamoto University (Kumamoto). Between October 2006 and May 2007, all consecutive patients who met the following criteria were enrolled. Eligible patients were 20 years of age or older and admitted within 7 d after the onset of acute ischaemic stroke, including cerebral infarction and transient ischaemic attack. Patients were excluded for any of the following: (i) active infectious endocarditis, (ii) urgent neurosurgery or cardiovascular surgery would be required, (iii) chronic thrombocytopenia (defined as a platelet count $<100 \times 10^9$/l for more than 30 d), (iv) haematopoietic malignancy and (v) an ongoing need for an anticancer-drug treatment. The study was approved by the research ethics committee of each centre. Heparin therapy was provided to a number of patients depending on the physician's decision (mainly considering the type of stroke and/or the patient's clinical status as described in the Introduction.)

Evaluation

The following patient characteristics were obtained: age, sex, height, body weight, body-mass index, modified Rankin Scale (mRS) score (van Swieten *et al*, 1988) before stroke onset, vascular risk factors (hypertension, diabetes mellitus, dyslipidaemia, current and past smoking habits, drinking habit, including occasional drinking), past history (autoimmune disease, haemodialysis, renal dysfunction, angina, myocardial infarction, cerebral infarction, transient ischaemic attack, pulmonary thromboembolism, extremity gangrene, amputation of an extremity, angiography, heparin exposure, surgical procedure and HIT), platelet counts, antiplatelet/anticoagulant drug use and blood transfusions. The timing and period of heparin administration (including heparin flushes), changes in platelet count, and alternative anticoagulant therapy for HIT (if given) were also examined. Other risk factors for stroke, such as emboligenic heart diseases including atrial fibrillation, were assessed based on the criteria from the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study (Adams *et al*, 1993). Based on the neurological, radiological, cardiological and haematological profiles, the stroke subtype was determined according to the TOAST subtype classification system by a consensus of stroke neurologists. The neurological severity of each patient was assessed by an experienced stroke neurologist according to the National Institutes of Health Stroke Scale (NIHSS) score (Lyden *et al*, 1994) on admission and discharge, and at 3 months after onset. Patient global outcome was also assessed with mRS (van Swieten *et al*, 1988).

Clinical evaluation. The clinical probability of HIT was assessed using the 4Ts scoring system (Warkentin & Heddle, 2003), which is composed of four clinical features that are

given scores of 0, 1, or 2; magnitude of thrombocytopenia; timing of platelet count fall (in relation to heparin therapy); thrombosis or other sequelae; and presence of other explanations for thrombocytopenia. The case reports of the patients, filled out by their physicians, were assessed independently in a blinded fashion by the external Data Assessment Committee, which consisted of two stroke neurologists, according to the 4Ts scoring system after the patient follow-up was completed. If the judgment was not concordant between the two stroke neurologists, they discussed the cases to reach a final consensus and decision. Based on the 4Ts score, the estimated pretest probabilities of HIT were categorized into three groups: low (0–3), intermediate (4–5) and high (6–8) scores. We diagnosed the patients with an intermediate or a high score as 'potential HIT' and those with a low score as 'clinical non-HIT'. These objective assessments for the clinical probability of HIT were done after the patient follow-up was completed as described above, so that no results influenced clinical management. Therefore, some patients were ultimately diagnosed as HIT even though the physicians in charge did not suspect HIT as described in details in the Results section.

Serological evaluation. Blood samples were collected from all patients on the first (to the third) and 14th (±4) hospital days to be tested for anti-PF4/heparin Abs using ELISA (Asserachrom HPIA; Diagnostica Stago, Asnieres, France). The assays were performed in a blinded fashion after patient follow-up was completed. ELISA was performed according to the manufacturer's instructions. The titres of the samples were expressed as values of optical density (OD). The result was considered positive when the titre was greater than the cut-off value, which was determined using the reference control for each kit. To confirm the diagnosis of HIT, SRA was measured for all patients with a positive ELISA and/or ≥4 points in the 4Ts scoring system ($n = 29$). In addition, samples from 39 patients selected randomly from among all the patients were tested by SRA as a control. Samples were measured as described elsewhere at the Platelet Immunology Laboratory, McMaster University (Hamilton, ON, Canada) blinded to all clinical, platelet count and serological data (Warkentin *et al*, 1992). Any sample that produced ≥10% mean serotonin release with <10% release in the presence of high heparin (at a final concentration of 100 u/ml) and the anti-FcγRIIa monoclonal antibody (IV.3) was considered SRA-positive.

Diagnosis

Based on the results of both the 4Ts clinical score and the serological assays, patients were categorized into four groups as follows: (i) definite HIT (4Ts score ≥4 points with positive results in both ELISA and SRA), (ii) possible HIT (4Ts score ≥4 points with positive result in either ELISA or SRA) and (iii) clinically suspected HIT (4Ts score ≥4 points with negative results in both ELISA and SRA), seropositive status (4Ts score <4 points with positive in both ELISA and SRA). The remaining patients were categorized as HIT unlikely.

Statistical analysis

The variables between the groups of patients treated with and without heparin were compared using Fisher's exact test and the Wilcoxon test. For NIHSS, the change, NIHSS score at discharge minus that at admission, was also determined. Statistical analyses were performed using SAS software version 9.1 (SAS Institute Inc, Cary, NC, USA).

Results

Patient characteristics

A total of 267 patients (mean age 71·7 years; 66·2% men), who were admitted to three stroke centres within 7 d after stroke onset during a 6-month period, were enrolled. Intravenous UFH was administered to 172 patients (64·4%: heparin group) (Fig 1). Male gender, atrial fibrillation, previous ischaemic heart disease, history of surgery using UFH, and history of intra-arterial catheter procedures were significantly more common in patients treated with than without UFH (Table IA). In regard to stroke subtype, large artery atherosclerosis and cardioembolism were more frequent in patients treated with UFH, and small vessel occlusion was more frequent in those without UFH treatment. There was no significant difference in the history of antiplatelet drug use before admission between the patients treated with (66 cases, 38·4%) and without UFH (32 cases, 33·7%) ($P = 0·508$) (Table IA). Both the NIHSS score at discharge (median, 2 vs. 1, $P = 0·020$) and mRS at 3 months after stroke onset (median, 2 vs. 1, $P < 0·001$) were higher in patients treated with UFH (Table IB).

The incidence of HIT

Anti-PF4/heparin Abs were detected at any time point in 22 patients (12·8%) in the heparin group and in 3 (3·2%) of 95 patients who did not receive intravenous UFH respectively (Fig 1), and the difference was significant ($P = 0·008$). Seven patients (4·1%) were diagnosed as having potential HIT according to the 4Ts score (≥4 points). All seven patients had intermediate scores. Among them, three showed positive results in both ELISA and SRA, to give an incidence of definite HIT of 1·7% [95% confidence interval (CI): 0·4–5·0]. Possible HIT, clinically suspected HIT, and seropositive status were 0%, 2·3% ($n = 4$), and 2·3% ($n = 4$), respectively (Fig 1). Of the 95 patients with a positive ELISA who did not receive heparin within 3 months before admission and/or during hospitalization, three were SRA-negative. The OD values of anti-PF4/heparin Abs detected by ELISA seemed a little higher in definite HIT patients than the seropositive status group, although statistical analysis was not performed because of the

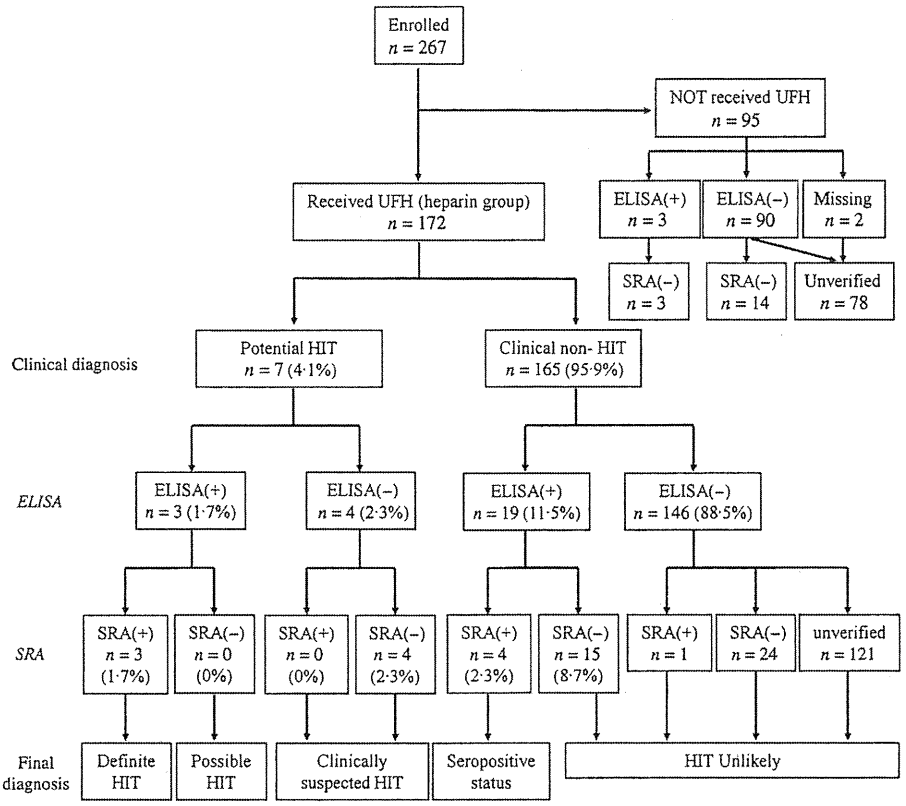

Fig 1. Flow chart for diagnosis of heparin-induced thrombocytopenia. HIT, heparin-induced thrombocytopenia; UFH, unfractionated heparin; ELISA, enzyme-linked immunosorbent assay; SRA, serotonin-release assay.

small sample size (Table II). OD values in ELISA did not correlate with the mean percentage release in SRA (Fig 2). However, the proportion of samples with positive-SRA to those with negative-SRA was greater in the samples with ≥1·5 OD value in ELISA as compared to those with <1·5 OD value. The prevalence of positive-ELISA was not significantly different between patients who received UFH for five or more days (15·9%) and for <5 d (11·4%).

Clinical course and the treatment of definite HIT patients

Only one (Case 3) of three definite HIT patients was suspected of having HIT by the treating physician. This patient had atrial fibrillation and an infarct in the right anterior and middle cerebral arteries. The admission NIHSS score was 17 (Table II). The patient's platelet count decreased from 156 to 99×10^9/l (approximately a 37% fall) in the typical HIT window (5–10 d) and recovered to 227×10^9/l soon after stopping heparin administration on day 7 due to the suspicion of HIT. The patient had a further fall in platelet count, from 227 to 99×10^9/l (approximately a 56% fall), after day 10 with a high OD value (2·086) in ELISA and a weak positive SRA (11% release) (Table II). The patient died due to deterioration from an underlying stroke. The very weak SRA, which was performed during the second platelet count fall, argues somewhat against this patient having HIT. However, HIT antibodies sometimes become weaker very quickly (Warkentin & Kelton, 2001; Greinacher *et al*, 2009), and so it is possible that the SRA would have been stronger during the first platelet count fall.

The other two patients (Cases 1 and 2) that ultimately met the criteria for definite HIT in this study were not suspected of having HIT by their physicians. One patient (Case 1) experienced a stroke of other determined aetiology due to arterial dissection in the intracranial left vertebral artery. The admission NIHSS score was 23 (Table II). The patient had bilateral cerebellar and brain stem infarcts. UFH was administrated for 7 d, and UFH flushes for intravascular catheter were continued for an additional 4 d. The patient showed a 52·0% decrease in platelet count, from 331 to 107×10^9/l, that began on day 5 of heparin with relatively high values in SRA (63·9% release) and ELISA (2·271 OD value) (Table II). Death occurred from stroke on day 11. The other patient (Case 2) with a previous history of recent transient ischaemic attacks had a cardioembolic stroke due to atrial fibrillation 9 d after urgent hemiarch replacement due to aortic dissection. The admission NIHSS score was 16. The patient's platelet count declined from 436 to 286×10^9/l (a drop of approximately 34%) during the typical HIT window of days 5–10 with relatively high values in SRA (51·6% release) and ELISA (1·725 OD value); although the platelet count evolution may be explained by a platelet count profile of post-cardiovascular surgery with cardiopulmonary bypass overshooting around postoperative day 14 and returning gradually to the baseline (Table II). The patient was dependent at discharge and at 3-month follow-up.

Table I. (A) Demographic data of patients treated or not with unfractionated heparin (UFH) and (B) clinical data of patients treated or not with UFH.

	With UFH (n = 172; 64·4%)	Without UFH (n = 95; 35·6%)	*P*-value
(A)			
Age (years), median (range)	71 (23–98)	73 (42–93)	0·515
Male gender (%)	122 (70·9)	53 (55·8)	0·015
Weight (kg)	60·1 ± 12·2	59·4 ± 11·6	0·673
BMI (kg/m^2)	23·3 ± 3·8	23·4 ± 3·7	0·936
HTN (%)	133 (77·3)	74 (77·9)	1·000
DM (%)	55 (32·0)	30 (31·6)	1·000
CRF (%)	17 (9·9)	5 (5·3)	0·247
HD (%)	3 (1·7)	0 (0)	0·555
Atrial fibrillation (%)	59 (34·3)	11 (11·6)	<0·001
Smoking (%)	78 (45·3)	37 (38·9)	0·303
Drinking (≥2 cups) (%)	49 (28·5)	21 (22·1)	0·249
Previous IHD (%)	33 (19·2)	5 (5·3)	0·002
Previous CVD (%)	51 (29·7)	28 (29·5)	1·000
Previous PTE (%)	0	0	–
Previous DVT (%)	4 (2·3)	1 (1·1)	0·658
History of heparin use within 3 months (%)	6 (3·5)	0 (0)	0·180
History of surgery using heparin	33 (19·2)	3 (3·2)	<0·001
History of intra-arterial catheter procedure (%)	43 (25·0)	8 (8·4)	<0·001
History of warfarin use (%)	18 (10·5)	5 (5·3)	0·176
History of antiplatelet agency use (%)	66 (38·4)	32 (33·7)	0·508
Stroke subtype			
TIA (%)	9 (5·2)	20 (21·1)	<0·001
Stroke (%)	163 (94·8)	75 (78·9)	
LAA (%)	38 (23·3)	5 (6·7)	
CE (%)	64 (39·3)	5 (6·7)	<0·001
SV (%)	26 (16·0)	48 (64·0)	
OT + UD (%)	35 (21·5)	17 (22·7)	
Platelet count (×10^9/l)	222 (103–583)	230 (119–483)	0·670
NIHSS score on admission, median (range)	5 (0–32)	3 (0–20)	<0·001
(B)			
Treatment during the hospital stay			
Warfarin use (%)	70 (40·7)	9 (9·5)	<0·001
Antiplatelet agency use (%)	105 (61·0)	84 (88·4)	<0·001
Cessation of heparin (%)	142 (82·6)	0	<0·001
Alternative anticoagulation (%)	67 (39·0)	37 (38·9)	1·000
Intra-arterial catheter procedure during the hospital stay (%)	70 (40·7)	0 (0)	<0·001
Surgery with heparin use during the hospital stay	7 (4·1)	0 (0)	0·053
Thromboembolic vents or death	25 (14·5)	4 (4·2)	0·012
Recurrence of ischaemic stroke	12 (7·0)	2 (2·1)	
Thromboembolic events during catheter	4 (2·3)	0	
Other thromboembolism	7 (4·1)	2 (2·1)	
React of heparin infusion	1 (0·6)	0	
Death	5 (2·9)	0	
NIHSS score at discharge, median (range)	2 (0–42)	1 (0–20)	–
NIHSS change, discharge-admission (range)	−2 (−21 to 19)	−1 (−8 to 9)	0·020
mRS at discharge, mean (median)	2 (0–6)	1 (0–5)	0·002
mRS at 3 months, median (range)	2 (0–6)	1 (0–5)	<0·001

BMI, body mass index; HTN, hypertension; DM, diabetes mellitus; CRF, chronic renal failure; HD, haemodyalysis; IHD, ischaemic heart disease; CVD, cerebrovascular disease; PTE, pulmonary thromboembolism; DVT, deep vein thrombosis; TIA, transient ischaemic attack; LAA, large artery atherosclerosis; CE, cardioembolism; SV, small vessel occlusion; OT, stroke with alternative aetiology; UD, stroke of undetermined aetiology; UFH, unfractionated heparin; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin scale.

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Table II. Clinical features of HIT patients.

Pt	Age (years)	Gender	Past history	Stroke subtype	4Ts score	ELISA (OD)	SRA (mean % release)	Platelet count ($\times 10^9$/l) Baseline	Nadir	Duration of UFH (day)	Duration of UFH up to the day of platelet nadir, days	Thrombotic complication	NIHSS on admission	mRS on discharge
Definite HIT														
1	62	Male	CI, HTN	Other	4	+(2·271)	+(63·9)	331	107	11	7	None	23	Dead
2	64	Female	CI, HTN, AF	CE	5	+(1·725)	+(51·6)	436	286	18	10	None	16	4
3	88	Female	AF	CE	5	+(2·086)	+(11·0)	156	99	7	15	None	17	Dead
Clinically suspected HIT														
4	67	Male	HTN, DM, AF, CRF	CE	4	−(0·138)	−(<1)	281	210	14	7	DVT	7	4
5	82	Male	CI, HTN, AF	CR	4	−(0·052)	−(<1)	137	27	1	4	None	10	4
6	66	Male	MI, HTN	CE	4	−(0·102)	−(<1)	583	225	13	17	None	12	1
7	69	Female	HTN, AF	CE	5	−(0·091)	−(<1)	297	120	23	6	RI	7	4
Seropositive status														
8	70	Female	HTN, AF	CE	0	+(1·666)*	+(53·2)	141	123	4	NA†	None	13	2
9	59	Female	HTN, AF, AID	CE	0	+(1·505)	+(76·8)	163	158	18	NA†	None	15	4
10	87	Male	IHD, HTN, AF	CE	0	+(0·977)	+(13·3)	200	150	13	NA†	None	8	5
11	90	Female	HTN, AF	CE	2	+(2·378)	+(28·8)	235	210	9	NA†	IHD	29	5

ELISA, enzyme-linked immunosorbent assay; SRA, serotonin-release assay; OD, optical density; CI, cerebral infarction; IHD, ischaemic heart disease; HTN, hypertension; DM, diabetes mellitus; AF, atrial fibrillation; CRF, chronic renal failure; MI, myocardial infarction within 4 weeks; AID, autoimmune disease; RI, renal infarction; DVT, deep vein thrombosis; other, stroke of other determined aetiology; CE, cardioembolism; NA, not applicable.

*ELISA was negative (OD: 0·079) in the sample drawn 7 d after admission, when SRA was positive. ELISA was positive (OD: 1·666) in the sample obtained 1 week later.

†Patient did not demonstrate thrombocytopenia.

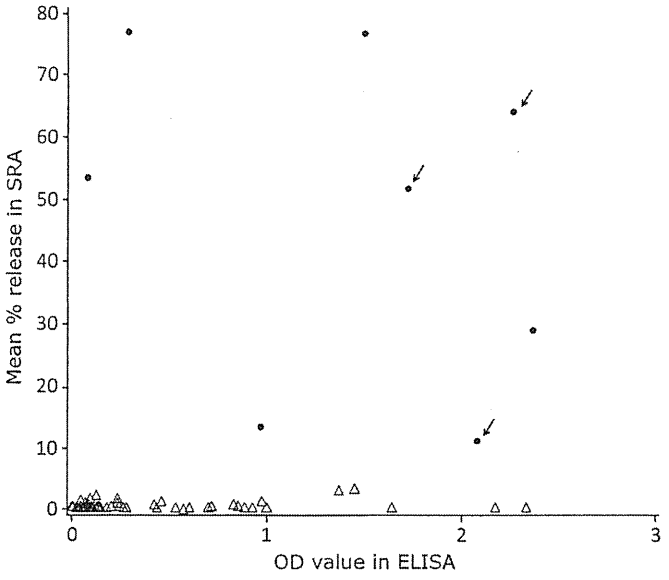

Fig 2. The correlation of optical density (OD) values for anti-platelet factor 4/heparin antibodies detected by enzyme-linked immunosorbent assay (ELISA) and mean percentage release by serotonin-release assay (SRA). These values showed poor correlation. Arrows indicate the data points of the three patients who met the criteria for definite HIT. •, SRA-positive cases, including one patient classed as 'HIT unlikely': OD = 0·298, and mean percentage release = 76·74; △, SRA-negative cases.

None of the patients in this study met the diagnosis of rapid or delayed onset HIT. None of the patients classified as definite HIT received treatment with alternative anticoagulants, such as thrombin inhibitors, nor did the patients develop additional thromboembolic events.

Discussion

HIT should be recognized as a clinicopathological syndrome because none of the currently available HIT diagnostic tools have sufficient sensitivity and specificity to be used as the primary or only tool to diagnose HIT. Thus, both clinical and serological diagnoses are crucial. In this prospective study, clinical probability was assessed using the 4Ts scoring system, which is a popular method, by two independent stroke neurologists who were blinded from the results of serological assays. As a result, 4·1% of the acute stroke patients treated with heparin were suspected clinically of having HIT with ≥4 points in the 4Ts scoring system. Among them, 1·7% (95% CI: 0·4–5·0) had platelet activating antibodies against the complexes of PF4 and heparin detected by ELISA and SRA, supporting the diagnosis of definite HIT. All of these definite HIT patients had intermediate scores in the 4Ts as well as four clinically suspected HIT cases, as shown in Table II. Thus, it was very difficult to distinguish HIT patients from non-HIT patients through clinical information alone. This may possible explain why only one among three definite HIT cases was suspected of having HIT by the treating physicians.

Our results were similar to those reported in other studies of patients with ischaemic stroke (Ramirez-Lassepas *et al*, 1984; Harbrecht *et al*, 2004) and the frequency of definite HIT was less than in surgical patients (Kappers-Klunne *et al*, 1997; Warkentin, 2007b). For two of the three definite HIT patients reported here, one had a possible alternative aetiology that could explain her platelet count fall (Case 2) and the other had a weak positive-SRA (Case 1) as described in detail in the Result section. Thus, we cannot exclude the possibility that these two patients might not have had HIT. If we exclude these patients, the incidence of HIT could be as low as 0·6%. However, this result was compatible with our previous retrospective study of the same patient population (the incident of HIT was 0·5%) (Kawano *et al*, 2008). Therefore, we can conclude that the incidence of HIT in acute stroke patients treated with UFH seems to be approximately 0·5–1·7%. These results emphasize that HIT diagnosis should be considered in the management of acute ischaemic stroke.

Another major finding was that the clinical severity and outcome of acute stroke patients who were diagnosed as having definite HIT were unfavourable. In particular, the in-hospital mortality of definite HIT was very high (66·7%). Previous reports also indicated that mortality was high in HIT patients (Warkentin *et al*, 1995, 2000; Kappers-Klunne *et al*, 1997). The present study is unique in that initial neurological severity and clinical outcomes of stroke patients with HIT were determined. The NIHSS score on admission (median, 17) in definite HIT was quite high, and the outcome at 90 d was poor. However, the poor outcome of those patients appeared to be mainly due to the severity of the initial stroke rather than HIT. Although clinical severity and outcome of patients treated with UFH were unfavourable compared to those without UFH, the patients with UFH intrinsically might be at high risk of thromboembolic complications because those patients more frequently had systemic atherosclerotic changes or embolic sources. In fact, stroke subtypes were distributed differently between patients with and without UFH in our study. Hoh *et al* (2005) reported significantly less favourable outcomes, including new thromboembolic episodes and deaths in patients with subarachnoid haemorrhage who developed HIT compared to those without HIT. They found that more patients with HIT showed a poorer Fisher Grade than those without HIT, although the diagnosis of HIT was based on clinical criteria, and serological examinations were not mandatory in the study (Hoh *et al*, 2005). It should be considered that serious neurological conditions might be vulnerable to HIT.

In the present study, four of 165 clinical non-HIT patients were positive by both ELISA and SRA. None of these patients demonstrated thrombocytopenia, nor did they die. A thromboembolic event occurred in one patient who developed an ischaemic heart event. Previous reports suggested that high OD values in ELISA and/or strong-positive SRA results were associated with a high degree of diagnostic accuracy for HIT (Warkentin *et al*, 1995, 2008; Lo *et al*, 2007). However, despite high OD values (≥1·5 units) in ELISA (Cases 8, 9, 11) or strong-positive (≥50% serotonin release) SRA results (Cases 8, 9), these patients did not develop HIT (Table II). One of the clinical non-HIT patients was ELISA-negative but SRA-positive and did not

develop any thrombocytopenia, thromboembolic event, or death. Furthermore, three of 95 patients without UFH were positive only by ELISA. In the present study, we blindly evaluated anti-PF4/heparin Abs in all clinical HIT and clinical non-HIT patients. Even if the results of anti-PF4/heparin Abs were positive, all patients with positive results would not always demonstrate HIT, and some of the positive results might not be pathological findings. Therefore, we should be aware of false negative and false positive results in both serological tests, and that diagnosis by the detection of anti-PF4/heparin Abs alone (even with a high OD value in ELISA and/or a strong-positive SRA result) can result in an overdiagnosis of HIT.

This study had some limitations. First, none of the patients underwent venous ultrasound; therefore, subclinical DVT, which is the typical thrombotic complication associated with HIT, may have been underdiagnosed. Second, the dose of UFH could be a determinant for the occurrence of HIT, as stoichiometrically optimal ratios of PF4:heparin influence immunization (Greinacher *et al*, 2008; Warkentin *et al*, 2010). However, in the present study, the dose and blood levels of UFH were not investigated.

In conclusion, the incidence of definite HIT in acute ischaemic stroke patients treated with UFH was 1·7% (95% CI: 0·4–5·0). HIT should be recognized as a clinicopathological syndrome in which both the clinical profile consistent with HIT and the results of serological tests should be carefully considered for HIT diagnosis. The clinical severity and outcome of acute stroke patients who were diagnosed as having definite HIT were unfavourable.

Author contribution

The study concept and design by H. Kawano, H. Yamamoto, S. Miyata, M. Izumi, and T. Hirano; writing by H. Kawano, H. Yamamoto, and S. Miyata; data collection by H. Kawano, H. Yamamoto, N. Toratani, M. Izumi, and T. Hirano; blinded independent assessments of the 4Ts score by S. Sato and S. Okamoto; ELISA assay by S. Miyata and I. Kakutani; SRA assay by Jo-AI. Sheppard and TE. Warkentin; analysis and interpretation of data by H. Kawano, H. Yamamoto, S. Miyata, and A. Kada; drafting of the manuscript by H. Kawano, H. Yamamoto, and S. Miyata; critical revision of the manuscript for important intellectual content by K. Toyoda, K. Nagatsuka, H. Naritomi, TE. Warkentin, and K. Minematsu; study supervision by M. Uchino and K. Minematsu.

Source of funding

Grant from the Bayer Scholarship for Cardiovascular Research, Japan Cardiovascular Research Foundation. Health and Labour Sciences Research Grant from the Japanese Ministry of Health, Labour and Welfare (Research on Clinical Trials' Infrastructure Development). Grant-in-Aid from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (06-51).

Disclosures

Dr Izumi, Dr Toratani, MT. Kakutani, MPH. Kada, Dr Sato, and Dr Okamoto report no disclosure. Dr Kawano received honoraria from Mitsubishi Tanabe Pharma Co. Ltd., for scientific lecture. Dr Yamamoto served on a scientific advisory board on Behlinger-Ingelheim, Data and Safety Monitoring Board of JASAP, scientific consultant for submission for drug of approval Mitsubishi Welpharma Co. Ltd, and received research grants as chief investigator from Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan; H18-Rinken-Wakate-005; and Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan; H19-Tokubetsu-Shitei-033, The Bayer Scholarship for Cardiovascular Research, Japan Cardiovascular Research Foundation, and Pfizer Health Research Foundation. Dr Miyata serves on the editorial advisory board for *Japanese Journal of Transfusion and Cell Therapy*, and *Japanese Journal of Thrombosis and Hemostasis*, received speaker's honoraria from Mitsubishi Tanabe Pharma Co. Ltd., Daiich Sankyo Co. Ltd., Sanofi Aventis, and GlaxoSmithKline, received research grants from Mitsubishi Tanabe Pharma Co. Ltd. and Daiichi Sankyo Co., Ltd, and research support from Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan (15C-1, 17C-7, H21-Iyaku-Ippan-005), Grant-in-Aid from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (06-51). Dr Hirano served as a imaging reading panel of J-ACT II sponsored by Mitsubishi Tanabe Pharma Co. Ltd. and Kyowa Hakko Kirin corporation, and received a research grant from The Ministry of Education and Science of Japan Scientific Research grant-in-aid, 50346996 as principle investigator. BSc. Sheppard supported from Heart and Stroke Foundation of Ontario – HSFO, T6157, for Research Assistant. Dr Warkentin receive royalties from publishing of Book: Heparin-Induced Thrombocytopenia; Publisher: Informa Healthcare USA, 2007, served as a scientific consultant for Canyon Pharmaceuticals; GTI Inc.: GlaxoSmithKline; Paringenix, served as a speaker's bureaus of GlaxoSmithKline; Pfizer Canada; Sanofi-Aventis, research supports form GlaxoSmithKline, GTI Inc, Heart and Stroke Foundation of Ontario; principal investigator; Grant Number T6157; and Heart and Stroke Foundation of Ontario grants NA6221 and T6763; co-investigator. Dr Nagatsuka received speaker's honoraria from Tanabe Mitsubishi Co. Ltd, Otsuka Pharmaceutical Co. Ltd, Daiichi-Sankyo Pharmaceutical Co. Ltd, Pfizer Japan Co. Ltd, research supports from Lundbeck Inc., Mitsubishi Tanabe Pharma Co. Ltd, and Ministry of Health, Labour and Welfare, H22-Junkanki-Ippan-006, 2010. Dr Naritomi received speaker's honoraria from Mitsubishi Tanabe Co. Ltd, Otsuka Pharmaceutical Co. Ltd, Kyorin Pharmaceutical Co. Ltd, and Kowa Co. Ltd. Dr Toyoda serves as an assistant editor of *Stroke*, received research grants from Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan; 20-Junkanki-Ippan-019; Chief investigator. Dr Uchino received honoraria from Mitsubishi Tanabe Pharma

Co. Ltd, Sanofi-aventis, Daiichi-Sankyo Co. Ltd., for scientific lecture, and received a research grant from Japanese Ministry of Education, Science, Sports and Culture/Grant-in aid for Scientific Research, 20591003, and Gene therapy of Duchenne muscular dystrophy. Dr Minematsu serves on the editorial boards of *Cerebrovascular Diseases*, the *International Journal of Stroke*, and the *Journal of Stroke and Cerebrovascular Diseases* and receives research support from Asteras Pharma Inc., Takeda Pharmaceutical Co. Ltd., Sanofi-Aventis, Lundbeck Inc., Mitsubishi Tanabe Pharma Co. Ltd., Kyowa Hakko Kirin Pharma, Inc., Hitachi Medical Corporation, MHLM, Japan, Research Grants for Cardiovascular Diseases, Grant-in-Aid, and the Foundation for Biomedical Research and Innovation, and honoraria from Daiichi Sankyo Co., Ltd.

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Carotid Duplex Ultrasonography Can Predict Outcome of Intravenous Alteplase Therapy for Hyperacute Stroke

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We evaluated whether carotid duplex ultrasonography (US) can help predict the safety and efficacy of treating hyperacute stroke with intravenous (IV) tissue plasminogen activator (alteplase) therapy. Consecutive patients with stroke were assigned to the carotid artery occlusion (CO) group or the other (non-CO) group according to US findings before or immediately after receiving IV alteplase. Effectiveness and safety outcomes included early neurologic improvement, defined as a reduction in a National Institutes of Health Stroke Scale (NIHSS) score of ≥4 points within the initial 24 hours after stroke onset; completely independent routine activity, defined as a modified Rankin Scale score of ≤1 at day 90 after stroke onset; symptomatic intracranial hemorrhage (ICH) occurring within 36 hours after stroke onset; and any ICH. We enrolled 127 patients (27 in the CO group and 100 in the non-CO group) with a median baseline NIHSS score of 13 (range, 4-30). The CO group had a higher baseline NIHSS score (median, 18 vs 12; $P = .005$). After multivariate adjustment, the CO group was inversely associated with early improvement (odds ratio [OR] = 0.26; 95% confidence interval [CI] = 0.09-0.72) and independence at day 90 (OR = 0.23; 95% CI = 0.05-0.73) and positively associated with any ICH (OR = 3.11; 95% CI = 1.23-8.48). Our findings indicate that CO identified by US in the emergency clinical setting is an independent predictor of unfavorable outcome and ICH following IV alteplase therapy. **Key Words:** Alteplase—internal carotid artery occlusion—intracranial hemorrhage—ultrasonography—outcome.

Occlusion of the internal carotid artery (ICA) often provokes severe hypoperfusion of cerebral blood flow in the affected territory. Patients who sustain acute ICA occlusion tend to have poor clinical outcomes.[1] Mortality is high in patients with malignant middle cerebral artery (MCA) infarction, resulting principally from distal ICA occlusion. The fates of patients with and without a major arterial occlusive lesion might differ after intravenous (IV) tissue plasminogen activator (alteplase) therapy, because resistance to clot lysis and the fragility of infarcted brain tissue may depend on the patency of the ICA. Rapid evaluation of arterial status in the emergency clinical setting may help predict outcome after alteplase therapy.

Magnetic resonance angiography (MRA) and computed tomographic angiography (CTA) can detect occlusions or severe stenoses of the cervicocephalic arteries supplying the infarcted area in patients with acute stroke,[2,3] as well as intracranial abnormalities with greater sensitivity and specificity, than conventional cerebral angiography.[3,4] Large ischemic lesions on diffusion magnetic resonance

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Received August 17, 2009; revision received September 25, 2009; accepted October 2, 2009.

Supported in part by a Grant-in-Aid (H20-Junkanki-Ippan-019) from the Ministry of Health, Labor, and Welfare of Japan.

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1052-3057/$ - see front matter

doi:10.1016/j.jstrokecerebrovasdis.2009.10.003

imaging (MRI) before IV alteplase therapy predict poor outcome in patients with acute ischemic stroke,[5] and diffusion–perfusion mismatch can select patients with remaining salvageable tissue.[6] But MRI takes at least 15 minutes, including equipment arrangement and patient transfer, to generate information, and CTA carries a risk of renal failure and anaphylaxis.

Carotid duplex ultrasonography (US) is another noninvasive tool that can detect major extracranial carotid arterial disease.[7-10] Compared with conventional cerebral angiography, US is not associated with such invasive complications as cerebral and systemic embolism, contrast agent anaphylaxis, acute renal dysfunction, and arterial dissection.[11] Morever, with bedside US, it takes only a few minutes to detect significant occlusive lesions of carotid arteries. US findings can help identify the mechanism and type of ischemic stroke.

We tested the hypothesis that carotid duplex US findings can help predict the outcome and safety of IV alteplase therapy for patients with hyperacute ischemic stroke.

Materials and Methods

We prospectively enrolled all patients with stroke who were admitted to our emergency stroke care unit and received IV alteplase therapy between October 2005 (when this therapy was approved in Japan) and July 2008. Our institution's Ethics Committee approved the research protocol. Patients or their representatives (eg, family members) provided written informed consent for the treatment.

Patient eligibility for IV alteplase therapy was based principally on the inclusion and exclusion criteria applied in the National Institute of Neurological Disorders and Stroke (NINDS) study[12] and in the Japan Alteplase Clinical Trial (J-ACT).[13] Each patient received a single IV dose of 0.6 mg/kg (not exceeding 60 mg) of alteplase, with 10% given as a bolus, followed by a continuous IV infusion of the remainder over 1 hour, in accordance with the Japanese guidelines for IV alteplase therapy based on the J-ACT results.[13,14] As in the NINDS study,[12] the use of antithrombotic agents were prohibited for 24 hours after onset, blood pressure was maintained at <180/105 mm Hg, and neurologic symptoms were monitored.

Clinical data included age and sex; time from symptom onset (or time when the patient last appeared to be normal) to the initiation of IV alteplase therapy; carotid artery US findings before or immediately after the initiation of alteplase therapy; National Institute of Health Stroke Scale (NIHSS) score immediately before (baseline) and 24 hours after alteplase therapy; concomitant diseases; current smoking and drinking habits; imaging data, including hemorrhagic transformation detected by computed tomography (CT) or MRI during hospitalization; stroke subtype according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria;[15] and modified Rankin Scale (mRS) score at day 90. Among concomitant diseases, hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg before stroke onset or the use of antihypertensive medication. Diabetes was defined as preceding fasting blood glucose ≥126 mg/dL or the use of oral antidiabetic agents or insulin. Hypercholesterolemia was defined as total plasma cholesterol level ≥220 mg/dL or the use of antihypercholesterolemic medication.

Patients underwent US after hospitalization while awaiting the results of blood tests or immediately after starting alteplase therapy. US was performed with a bedside unit (Sonos 5500; Philips Medical Systems, Tokyo, Japan) with a 3- to 11-MHz linear transducer. On US, absent color flow signals on the ICA indicates the occlusion at or proximal to the artery, and absent end-diastolic flow velocity of the ICA indicates intracranial ICA occlusion.[16] Thus, carotid artery occlusion was defined as either of these US findings (Fig 1). Based on the US findings, the patients were divided into 2 groups: those with carotid artery occlusion (designated the CO group) and those without carotid artery occlusion (designated the non-CO group).

Before alteplase therapy, all patients underwent intracranial MRA to serve as the gold standard reference of carotid US findings, unless contraindicated. MRA was performed using the 3-dimensional time-of-flight technique (repetition time/echo time, 35/7.2 msec; 20-degree flip angle) with a 1.5 T system (Magnetom Vision; Siemens, Germany).

Outcomes included early neurologic improvement, defined as a ≥4-point reduction in NIHSS score within the initial 24 hours, and complete independence in activities of daily living (ADL), defined as an mRS score of 0 or 1, at 90 days. To assess long-term independence, patients with a mRS score of ≥2 before stroke onset were excluded. Safety outcomes included any intracranial hemorrhage (ICH) confirmed by head CT or MRI during hospitalization, and symptomatic ICH defined as early ICH with neurologic deterioration corresponding to a ≥1-point increase in the NIHSS score within 36 hours after alteplase therapy.

Statistical Analysis

Sensitivity, specificity, positive predictive value, and negative predictive value for detectng patients with carotid artery occlusion by carotid US were calculated when intracranial MRA findings were used as gold standard. Continuous and categorized variables were compared using the Student *t*-test and the χ^2 test, respectively. Nonparametric independent group comparisons were done using the Mann-Whitney *U*-test. To determine independent clinical variables to predict outcomes, significant variables were analyzed in a logistic regression model, with multivariate adjustments for age, sex, and confounders with an association of $P < .05$ with each outcome in univariate analysis. Statistical significance was established at $P < .05$.

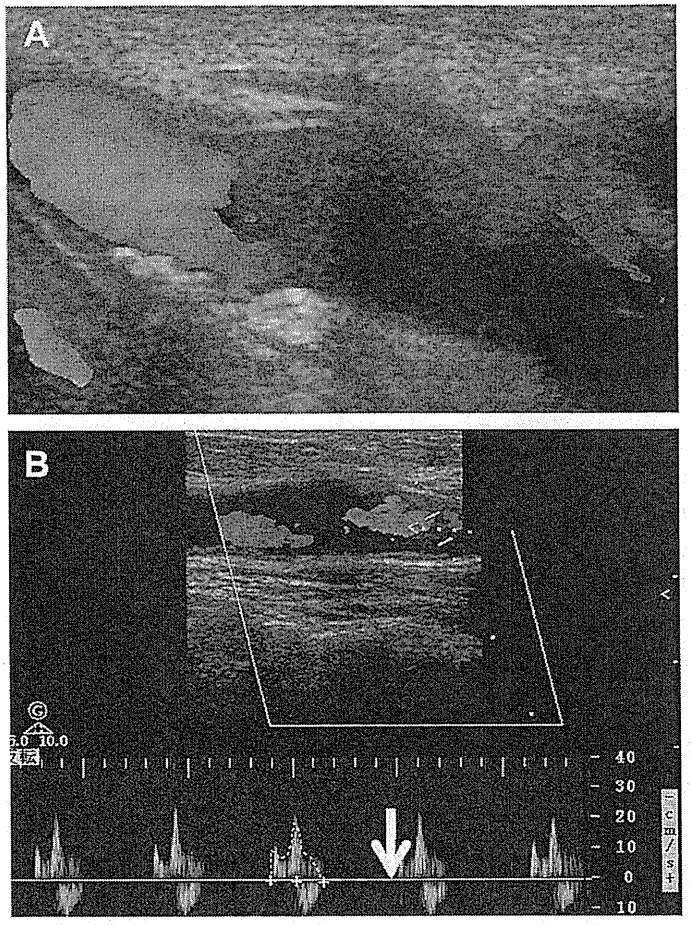

Figure 1. *Typical carotid US findings in ICA occlusion. (A) Absent flow of color in the affected ICA origin in a patient with atherothrombotic extracranial ICA occlusion. (B) Absent end-diastolic flow velocity of affected ICA (arrow) detected by pulsed Doppler US in a patient with distal ICA occlusion.*

Results

A total of 127 patients (89 men, mean age, 73 ± 9 years) were enrolled in the study. In 27 patients, carotid artery occlusion was detected by carotid US before or immediately after alteplase therapy. A total of 110 patients (87%) underwent MRA; 23 were found to have ICA occlusion. Sensitivity, specificity, positive predictive value, and negative predictive value for detect carotid artery occlusion by carotid US were 96%, 97%, 88%, and 99%, respectively. Table 1 summarizes the baseline characteristics and clinical outcomes of the study population. The median baseline NIHSS score was 13 (range, 4-30) and was higher in the CO group than in the non-CO group ($P = .005$). The median duration from symptom onset to IV alteplase therapy was 135 min (range, 50-180 min). US found no evidence of common carotid artery dissection possibly extending from the aortic arch in any patient. This finding, in combination with later examinations, ruled out aortic dissection in all patients.

Cardioembolism was the leading stroke subtype (57%). Atrial fibrillation was more common in the CO group than in the non-CO group. Early neurologic improvement and independence at day 90 were apparently less frequent in the CO group, whereas any ICH was more frequent in the CO group. Two patients in the CO group (7.4%) died within 90 days, one of symptomatic ICH and the other (who had asymptomatic ICH) of severe cerebral herniation due to massive stroke.

We used univariate analysis to test associations of the characteristic variables listed in Table 1 with outcomes (Table 2). Baseline NIHSS ($P = .042$), diabetes mellitus ($P = .049$), and carotid artery occlusion ($P = .039$) were inversely associated with early neurologic improvement. High pretreatment NIHSS score ($P = .015$) and carotid artery occlusion ($P = .002$) were inversely associated with independence at day 90. High baseline NIHSS score ($P = .047$) and carotid artery occlusion ($P = .009$) were associated with any ICH. No variables were significantly associated with symptomatic ICH.

We analyzed the contributing factors to the efficacy and safety outcomes using multivariate adjustment (Table 3). The CO group was independently associated with the absence of early neurologic improvement (odds ratio [OR] = 3.79; 95% confidence interval [CI] = 1.39-11.42; $P = .008$), absence of complete independence at day 90 (mRS score of ≥2: OR = 4.44; 95% CI = 1.38-19.96; $P = .011$), and presence of ICH (OR = 3.11; 95% CI = 1.23-8.48; $P = .016$). Diabetes mellitus (OR = 2.77; 95% CI = 1.03-8.15; $P = .043$) and low NIHSS score (OR = 1.09; 95% CI = 1.02-1.18 per 1-point decrease; $P = .011$) were associated with the absence of early neurologic improvement.

Discussion

Our data indicate that the likelihood of a good outcome was decreased and the likelihood of ICH was increased in stroke patients with US-identified ICA occlusion after IV alteplase therapy. Rapid evaluation using US thus helped predict the effectiveness and safety of alteplase therapy.

Sites of arterial occlusion before alteplase therapy have frequently been identified using transcranial Doppler (TCD) sonography. Recanalization of the ICA after IV alteplase therapy documented on TCD or angiography is reportedly complete in 10% of patients, partial in 16%, and absent in 74%.[17] In addition, terminal ICA occlusion has the least likelihood of recanalization compared with the other types of occlusion (OR = 0.1).[18] Linfante et al[19] found that patients with ICA occlusion have higher NIHSS scores on days 1 and 3 and a lower proportion of recanalization defined by TCD or MRA compared with those with MCA occlusion after alteplase therapy. Consequently, occlusions at the terminal ICA and at a tandem lesion of the ICA and MCA are predictive of poor outcome after alteplase therapy.[18,20] On the other hand, whether carotid US can detect ICA occlusion in the clinical setting of alteplase therapy has not been unequivocally established.

We used carotid US to evaluate the major cerebral arteries because Asian patients with stroke generally do

Table 1. *Baseline characteristics and clinical outcomes*

		US findings	
	Total (n = 127)	CO group (n = 27)	Non-CO group (n = 100)
Characteristic variables			
Female sex	38 (30)	8 (30)	30 (30)
Age, years	73 ± 9	75 ± 8	73 ± 10
Baseline NIHSS score	13 (4-30)	18 (5-24)	12 (4-30)§
Onset to treatment, minutes	135 (50-180)	130 (79-180)	135.5 (50-180)
Hypertension	80 (64)	21 (78)	59 (59)
Diabetes mellitus	24 (19)	5 (19)	19 (19)
Hypercholesterolemia	34 (27)	7 (26)	27 (27)
Atrial fibrillation	58 (46)	17 (63)	41 (41)‡
Current smoking	31 (25)	8 (30)	23 (23)
Alcohol	59 (47)	14 (52)	45 (45)
Stroke subtype			
Large vessel	21 (17)	7 (26)	14 (14)
Cardioembolic	72 (57)	16 (59)	56 (56)
Small vessel	2 (2)	0 (0)	2 (2)
Other	32 (26)	4 (15)	28 (28)
Outcome variables			
Early neurologic improvement*	60 (47)	8 (30)	52 (52)‡
mRS score at 3 months	3 (0-6)	4 (0-6)	2 (0-6)§
Complete independence at 3 months†	44 (35)	3 (11)	41 (41)§
Any intracranial hemorrhage	61 (48)	19 (70)	42 (42)§
Symptomatic intracranial hemorrhage	5 (4)	1 (4)	4 (4)

Values are mean ± standard deviation in age, median (range) in baseline NIHSS score, interval between onset and treatment and mRS score at 3 months, or number (%) in the remaining variables.

*Reduction in NIHSS score of ≥4 points within the initial 24 hours.

†Defined as a mRS score of 0 or 1. Eleven patients with a score ≥2 before stroke onset were excluded.

‡$P < .05$.

§$P < .01$.

not have a sufficient bone window for TCD,[21,22] and obtaining information about arterial occlusion from TCD can be difficult. As an alternative, carotid US can detect intracranial ICA occlusion based on the absence of end-diastolic flow velocity.[16] The accuracy of the diagnosis of carotid occlusion by US is sufficiently high compared with MRA findings. B-mode, color Doppler, and pulsed-wave Doppler carotid US can identify an ICA occlusion in about 5 minutes. The American Heart and Stroke Association recommends completing the initial evaluation and starting medical therapy within 60 minutes of the patient's arrival at the emergency department.[23] Head CT and bedside carotid US imaging can be completed at the emergency department within the 20 minutes or so needed to generate the results of blood tests, including serum chemistry and hemostatic parameters, at our institute.

Another reason for the routine use of carotid US is to rule out aortic dissection extending to the CCA. Concomitant aortic dissection is a conspicuous cause of in-hospital death following IV alteplase therapy in Japan (Japan Stroke Society; http://www.jsts.gr.jp [in Japanese]).

The present study has some limitations. Carotid US cannot provide information about tandem lesions. The incidence of symptomatic ICH was too low to enable an assessment of its relationship with carotid US findings.

In summary, carotid US is a simple tool for detecting ICA occlusion within a few minutes in the emergency clinical setting of hyperacute stroke. Patients with ICA occlusion according to carotid US had worse outcomes and more ICH after IV alteplase therapy. Therefore, rapid non-invasive evaluation of the carotid artery using US might improve the selection of patients likely to benefit from IV alteplase therapy. Although ICA occlusion is a pessimistic sign for success in IV alteplase therapy, patients with such a lesion may still be candidates for this therapy until an alternative therapeutic strategy is established. In the near future, endovascular thrombus retrieval and